Isolates of Zaire ebolavirus from wild apes reveal genetic lineage and recombinants

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Over the last 30 years, Zaire ebolavirus (ZEBOV), a virus highly pathogenic for humans and wild apes, has emerged repeatedly in Central Africa. Thus far, only a few virus isolates have been characterized genetically, all belonging to a single genetic lineage and originating exclusively from infected human patients. Here, we describe the first ZEBOV sequences isolated from great ape carcasses in the Gabon/Congo region that belong to a previously unrecognized genetic lineage. According to our estimates, this lineage, which we also encountered in the two most recent human outbreaks in the Republic of the Congo in 2003 and 2005, diverged from the previously known viruses around the time of the first documented human outbreak in 1976. These results suggest that virus spillover from the reservoir has occurred more than once, as predicted by the multiple emergence hypothesis. However, the young age of both ZEBOV lineages and the spatial and temporal sequence of outbreaks remain at odds with the idea that the virus simply emerged from a long-established and widespread reservoir population. Based on data from two ZEBOV genes, we also demonstrate, within the family *Filoviridae*, recombination between the two lineages. According to our estimates, this event took place between 1996 and 2001 and gave rise to a group of recombinant viruses that were responsible for a series of outbreaks in 2001–2003. The potential for recombination adds an additional level of complexity to unraveling and potentially controlling the emergence of ZEBOV in humans and wildlife species.

phylogeny | emergence | *Filoviridae* | nucleoprotein | glycoprotein

Among the recognized species of *ebolavirus* (family *Filoviridae*), the Zaire ebolavirus (ZEBOV) is the most virulent, causing acute hemorrhagic fever that can lead to death in a matter of days, with case fatality rates ≤88%. Only ≈10 recognized ZEBOV outbreaks among humans have occurred to date, and all of these took place in west-central Africa during three distinct periods: 1976–1977, 1994–1997, and 2001–2005 (1). Outbreaks have been shown to coincide with significant mortality among wildlife in surrounding areas (2, 3), and it is thought that people first acquire infection through the handling of infected animal carcasses (1, 4, 5). Ecological survey data indicate that the detrimental effects of ZEBOV infection on wildlife populations, mainly chimpanzees and gorillas, over the last two decades have been much more dramatic and widespread than the frequency and geographic scale of human outbreaks alone would suggest (2, 6–8).

Despite numerous studies of human and animal infection, attempts to reconstruct the history and evolution of ZEBOV have been hindered by the small number of isolates available. Only 12 glycoprotein (*GP*) gene sequences have been published to date, and all correspond to human isolates recovered between 1976 and 2002. Phylogenetic analysis shows that these sequences belong to a single lineage with time-dependent evolutionary changes. This tree topology, combined with the temporal-spatial pattern of ZEBOV emergence over the last two decades, led some authors to suggest that outbreaks are epidemiologically linked and occur at the front of an advancing wave that emerged *ca.* 1976 in the Democratic Republic of the Congo (9). However, the identification of multiple strains during the 2001 Gabon/Republic of the Congo (RC) outbreak led other authors to deduce that ZEBOV repeatedly and independently spilled over from the reservoir species (most likely bats) (10) into wildlife and human populations (3). In this multiemergence hypothesis, Ebola outbreaks would occur episodically in certain ecological conditions caused by habitat disturbances or climatic phenomena (11, 12). Further implicit is the idea that ZEBOV was present in the affected area of Central Africa long before the first documented outbreak in 1976.

Given the constant risk of new human outbreaks and the disastrous consequences that outbreaks mean for endangered great ape populations, there is an urgent need to test the competing models of ZEBOV emergence against more data to develop better predictions and potential control strategies. Arguably the most important piece of information missing in our understanding of ZEBOV epidemiology and evolution is a characterization of the viral strains circulating among susceptible wildlife species. Based on a unique data set collected during a 5-year period, we describe the first isolates of ZEBOV from great ape carcasses in the Gabon/RC region, as well as two recent human outbreaks. We also provide additional sequence data from previous outbreaks. Together these data permit a critical reevaluation of the existing models of ZEBOV emergence and shed new light on the evolutionary dynamics of the virus.

Results

Between 2001 and 2006, we discovered 47 dead animals, of which 23 were great apes (18 gorillas, 5 chimpanzees), in the Gabon/RC

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Abbreviations: CP, codon position; ML, maximum likelihood; RC, Republic of the Congo; ZEBOV, Zaire ebolavirus.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. EU051634 and EU051647 (Etoumbi_may05), EU051635 and EU051648 (Mbandza_nov03), EU051630 and EU051649 (GOR1_Lossi_dec02), EU051631 (GOR2_Lossi_dec02), EU051632 (GOR_Ekata_nov01), EU051633 (CH_Lossi_feb03), EU051636 (GOR_Mbandza_jun03), EU051637 (GOR1_odz_june05), EU051638 and EU051650 (GOR_odz_jun05), EU51639 (Mayibout_96), EU05164 (Mendemba_A_oct01), EU051641 (Mendemba_B_oct01) EU051642 (Etakangaye_dec01), EU051643 (Olloba_dec01), EU051645 (Mvoula_jan03), EU051644 (Entsiami_jan03), and EU051646 (Yembelengoye_jan03)].

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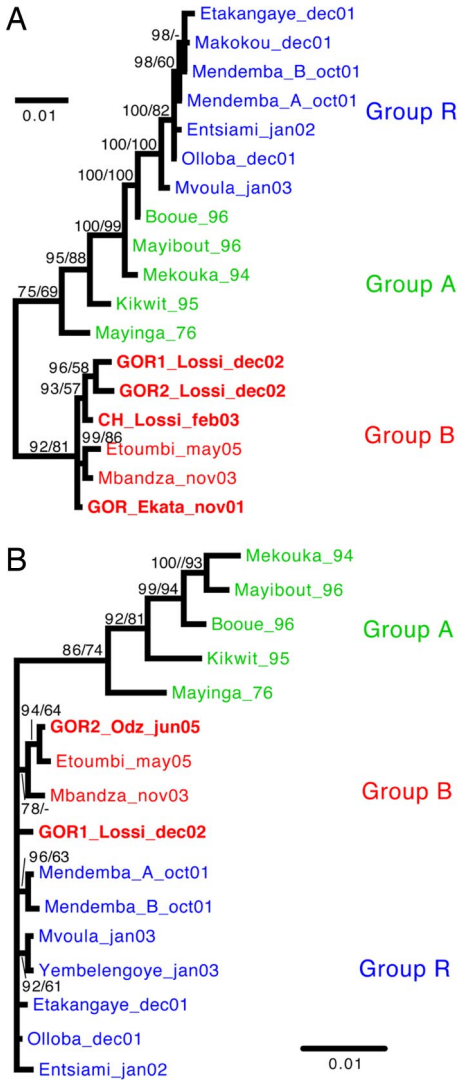

Fig. 1. Phylogenetic trees inferred from *GP* gene (*A*) and *NP* gene (*B*) sequences that show relationships among all of the ZEBOV isolates. Viruses derived from wild ape carcasses are shown in bold. Values above branches represent Bayesian posterior probabilities and ML bootstrap scores (both as percentages). Colors identify three genetic groups of ZEBOV: green, group A; red, group B; blue, recombinant group R.

region [supporting information (SI) Table 2]. Using a combination of diagnostic tests, we confirmed infection with ZEBOV for 13 gorillas, 3 chimpanzees, and 1 duiker. Because of the advanced decomposition of several carcasses, we only succeeded in amplifying and sequencing full-length *GP* gene sequences [2,031 nucleotides (nt)] from three gorillas and one chimpanzee, but shorter *GP* sequences (538 nt) could be obtained from another three gorillas. Phylogenetic analysis of the full *GP* revealed that these ape-derived sequences represent a new, distinct ZEBOV lineage (group B) that is 2–3% divergent from the lineage containing all *GP* sequences described to date (groups A and R) (Fig. 1*A*). Analysis of the partial *GP* data set containing the additional three sequences from gorillas confirmed this result (SI Fig. 4). In addition to the virus from ape carcasses, *GP* sequences generated from the two most recent human outbreaks in Mbandza (2003) and Etoumbi (2005) in RC also fell into the new group B (Fig. 1*A*).

Three full *GP* sequences that originated from ape carcasses located in the Lossi sanctuary in RC (GOR1_Lossi_dec02, GOR2_Lossi_dec02, and CH_Lossi_feb03) grouped together in the phylogeny, with the two sequences from gorillas clustering most closely (Fig. 1*A*). In December 2002, two gorilla carcasses were found together, a few meters from each other and in the same state of decomposition, indicating that they were infected and died around the same time. They probably belonged to the same group because a single group of gorillas was known to inhabit this part of the sanctuary. Despite this finding, *GP* sequences from these carcasses differed substantially at both the nucleotide and amino acid levels (11 nt and 5 aa differences) (SI Table 3), suggesting that they had not been infected by the same source.

We also obtained partial sequence data (1,448 nt) from the nucleoprotein (*NP*) gene from all human outbreaks since 2001, including the most recent ones in Mbandza and Etoumbi, as well as from two gorilla carcasses discovered in the Lossi and Odzala sanctuaries. Consistent with results for *GP*, sequences from apes and the two recent human outbreaks clustered together (group B) and were genetically distinct from viruses found in the 1976–1997 outbreaks (group A) (Fig. 2*B*). Similar to *GP*, both groups were ≈2% divergent. However, the phylogenetic position of viruses detected in October 2001 to January 2003 differed markedly for the two tree topologies: Whereas for *GP*, these viruses fell into group A and represented direct descendents of viruses seen during previous outbreaks (Fig. 1*A*), their *NP* sequences clustered in a more basal position in the tree together with group B viruses, from which they were not genetically distinct (Fig. 1*B*). This result was highly suggestive of a recombination event between group A and group B viruses, which both occupied stable positions in the *GP* and *NP* trees. To further examine the evidence for recombination, we statistically evaluated the observed level of incongruence between the two tree topologies. Shimodaira–Hasegawa tests identified the *GP* and *NP* topologies as significantly incongruent regardless of how the comparison was made (in all cases, $P < 0.001$) (see *Materials and Methods*).

A molecular clock-based analysis estimated that the root of all ZEBOV sequences just barely predated the first observed outbreak in 1976. Estimates based on *GP* sequences placed this most recent common ancestor at 1975 (highest posterior density interval: 1972–1976), whereas *NP* data produced a similar point

Table 1. Estimated ages of critical nodes, representing the most recent common ancestors for certain groups of sequences, in the ZEBOV phylogenies (see Fig. 1)

	Glycoprotein (*GP*)			Nucleoprotein (*NP*)		
MRCA	Date	Upper	Lower	Date	Upper	Lower
Groups A and B	Sep. 1975	May 1972	Sep. 1976	Nov. 1971	March 1955	Aug. 1976
Group B	Jan. 2000	March 1998	April 2001	Dec. 1998	April 1995	Dec. 2000
Group R	Sep. 1999	May 1998	Oct. 2000		Same as group B	
Lossi gorillas	July 2001	March 2000	April 2002	ND	ND	ND

Dates were estimated for two viral genes using a Bayesian molecular clock-based approach. MRCA, most recent common ancestor; Date, median date associated with the MRCA; Upper/Lower, upper/lower 95% highest posterior density interval; ND, no data.

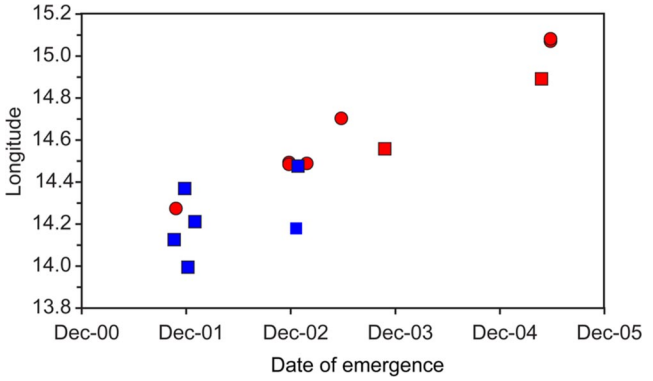

Fig. 2. Timing and longitudinal location of all ZEBOV outbreaks from 2001 onward that were included in the phylogenetic analysis. Blue and red symbols indicate outbreaks caused by group B and group R viruses, respectively. Squares, human outbreaks; circles, ape carcasses.

estimate of 1971, but with a wider interval (1955–1976). Ancestors of groups B and R, as well as those of the viruses from two Lossi gorillas, were estimated to be of an even younger age (Table 1).

Plotting the month of emergence for all ZEBOV isolates since 2001 included in the genetic analysis against their longitudinal distribution revealed a positive relationship, consistent with outbreaks spreading east at a fairly regular rate, regardless of the genetic type of virus involved ($R^2 = 0.859$, $P < 0.001$) (Fig. 2).

Discussion

Up to now, our understanding of ZEBOV epidemiology and evolution has been exclusively based on molecular data from human infections and largely involved only a single viral gene (*GP*). By presenting new genetic data from infected ape carcasses and recent human outbreaks for the *GP* and *NP* genes, we are able to provide a more complete, but also more complex, picture of the history and conditions of ZEBOV emergence in central Africa.

The existence of additional ZEBOV lineages in the African rainforests, other than those known from previous human outbreaks, has been suspected for some time. Although IgG prevalence rates in some great ape (5) and human populations with no history of hemorrhagic fever (2, 13) suggested the presence of apathogenic lineages, our data demonstrate the existence of another ZEBOV lineage capable of causing significant mortality in human and wildlife populations. Spatially, this new genetic group has so far only been found in a small area that shows almost no overlap with other groups, but that is situated immediately to the east of the area affected by the group R viruses a few years earlier (Fig. 3). In fact, ignoring the molecular data and considering solely the temporal and spatial sequence of outbreaks between 2001 and 2005, the pattern appears highly consistent with ZEBOV continuously spreading eastward (Fig. 2). However, our new genetic data reveal that the more recent outbreaks are not caused by genetic descendents of previously emerging viruses, as seen in previous data, but instead involve a genetically different type of ZEBOV. Thus, because outbreaks do not appear to be epidemiologically linked in a consistent fashion, these findings challenge the notion of ZEBOV emergence in recent years being driven by a single viral lineage spreading through the affected area of Gabon/RC (9).

A key aspect of ZEBOV epidemiology that is still poorly understood is the role and extent of animal–animal transmission among susceptible species like gorillas and chimpanzees (8). Recent data from habituated gorillas in the Lossi sanctuary that experienced mass mortality due to ZEBOV strongly suggest

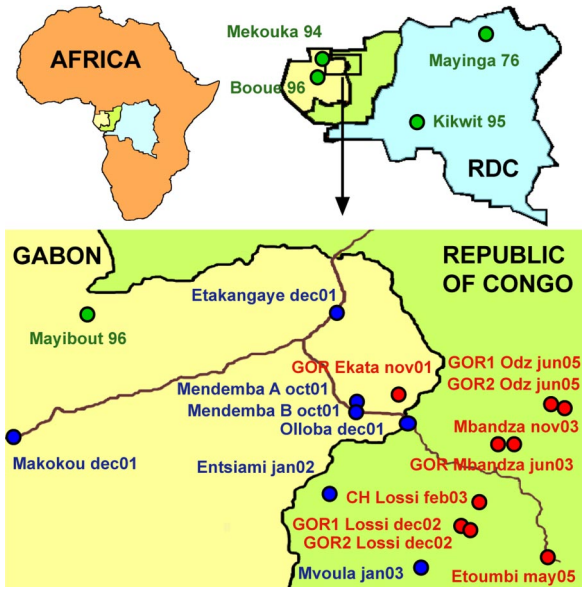

Fig. 3. Location of sources of all ZEBOV isolates genetically identified in humans and great apes from the first 1976 outbreak to the present. The source (GOR, gorilla; CH, chimpanzee), location, and month of sampling are specified. Colors relect virus groups (Fig. 1).

efficient transmission within family groups, as well as repeated spread to neighboring groups (6). If these gorillas indeed acquired infection predominantly from contact with infected conspecifics, diseased individuals from one group or locality would be expected to harbor highly related viruses. For example, several studies have found no genetic changes during chains of transmission involving human–human passage of ebolavirus for several weeks or months (14, 15). In contrast, we found that the two gorillas from Lossi, which were found next to each other in December 2002 and were likely infected and died around the same time, yielded viruses for which *GP* gene sequences differed at 11 sites (SI Table 3). Our molecular clock-based analysis indicates that this level of divergence implies independent evolution for a minimum of 8 months (Table 1). Thus, it seems most likely that the two gorillas became infected from different sources. Both sequences tightly grouped with each other and with a third Lossi sequence from a chimpanzee, still suggesting that all three animals were infected by the same local ZEBOV variant (Fig. 1*A*). Whether divergence among sequences reflects variability of a local virus in its reservoir or whether these changes accumulated during prolonged animal–animal transmission remains unresolved.

Results discussed so far point to a multiple independent emergence of ZEBOV in the Gabon/RC border area over the last decade. However, in line with previous studies (9, 16), we also continued to find low divergence among viral sequences, implying a recent common evolutionary origin for all ZEBOV lineages discovered so far. Despite the new group B viruses being clearly distinct from the longer known group A viruses, the two lineages are estimated to have split only in the early 1970s and thus just before the first recognized outbreak of ZEBOV in 1976, which was in the Democratic Republic of the Congo, 2,000 km from the Gabon/RC region. Similarly, the common ancestor of all group B viruses is estimated to date back no further than 1998 (Table 1). These dates are clearly at odds with the virus being a long-term resident in the affected area while being maintained by a widespread reservoir, mostly likely some fruit bat species: *Hypsignathus monstrosus*, *Epomops franquetti*, and *Myonycteris torquata* (1, 10).

Furthermore, it remains suspicious that groups A and B viruses emerged in such direct temporal and spatial succession and that the distribution of outbreaks from either type exhibited the same pattern consistent with eastward spread (Fig. 2). This finding suggests the existence of some underlying ecological factor that connects all outbreaks regardless of the genetic type of virus involved. Movement and contact processes among animals involving either reservoir or susceptible species, or both, may be potential mechanisms for this (8, 9).

Our comparative phylogenetic analysis of two viral genes produced a strong indication that a recombination event has occurred in the recent evolutionary history of ZEBOV (Fig. 1). All *GP* sequences from the putative recombinants are estimated to have shared a common ancestor in 1999 (Table 1), suggesting that a single incident gave rise to the recombinant lineage around this time. Where this event took place, in terms of both the geographic context and the host species, and how recombination affected viral fitness cannot be discerned at this point. However, it is interesting to note that all of the recombinant *GP* sequences contained a stop codon in amino acid position 481, which would be predicted to result in a 29% shorter translated protein product. Despite a truncated *GP*, the virus obviously remained viable, because members of this group were involved in a series of outbreaks. In future research, we hope to address the potential fitness benefits (or costs) of recombination in ZEBOV, as well as to describe the molecular mechanisms underlying it. Our finding represents the first evidence of a recombination event within the *Filoviridae* family (Ebola and Marburg viruses) and one of only a few examples of such an event in negative-strand RNA viruses (17). This result has important implications for vaccine development (18, 19) because it raises the possibility of recombination between a live attenuated vaccine strain and wild-type strains, a scenario that could have grave public health repercussions.

In conclusion, our results demonstrate that ZEBOV was likely introduced into susceptible wildlife and human populations repeatedly in the last 30 years, as evidenced by the emergence of different genetic lineages. What remains unclear is how often such introductions have occurred and to what extent transmission among susceptible animal hosts, such as great apes, may have subsequently contributed to the spatial propagation of outbreaks. Similarly, it remains to be resolved whether the temporal-spatial patterns of ZEBOV emergence, which are in many ways suggestive of a spreading process, could be the result of transmission processes in the reservoir species or whether other factors could have generated such patterns. In addition, our study reveals an unanticipated role of recombination in ZEBOV evolution. Besides raising a number of important questions about the biology of the virus, recombination is likely to further complicate efforts to understand the history of ZEBOV emergence by using molecular epidemiological approaches.

Materials and Methods

Investigations of Animal Mortality. Animal carcasses were found by ourselves and/or local villagers in the forested areas of northeastern Gabon and northwestern RC (Gabon/RC) during human Ebola outbreaks occurring between 2001 and 2005. Dead animals were rarely seen in the forest owing to their rapid decomposition and the small number of nonpredatory deaths. Over the 5-year study period, we found and tested 47 animal carcasses in the Gabon/RC human outbreak region. Necropsy specimens (skin, muscle, liver, or bone) were either placed in cryovials and immediately frozen in dry nitrogen or placed at room temperature in vials containing RNA Later solution (Qiagen, Valencia, CA). Laboratory tests were performed at the Centre International de Recherches Médicales de Franceville, Gabon. Tissues were tested for ZEBOV in the high-level security laboratory at the Centre International de Recherches Médicales de Franceville, using RT-PCR and antigen capture assays as described in ref. 5.

Investigations of Human Outbreaks. The two most recent ZEBOV outbreaks occurred in RC (20). One originated in Mbandza and was associated with 35 human cases (29 deaths) between October and December 2003, and the other originated in Etoumbi, where 12 human cases (9 deaths) were reported between April and June 2005 (Fig. 3). Blood samples from acutely ill patients from Mbandza ($n = 10$) and Etoumbi ($n = 1$) were collected on EDTA with the patients' oral informed consent, adhering to World Health Organization guidelines on viral hemorrhagic fever agents in Africa.

Reverse Transcription, DNA Amplification, and Nucleotide Sequencing. Samples of animal carcasses were fragmented and homogenized in the lysis buffer supplied with the RNeasy kit (Qiagen). Total RNA was extracted from the final supernatant with the RNeasy kit. For human samples, total RNA was extracted from plasma with the QIAamp viral RNA kit (Qiagen). First-strand cDNA was synthesized and then submitted to amplification with the primers and conditions described below. The amplicons were sequenced by GeneCust (Evry, France).

We attempted to amplify fragments of the ORF of the *GP* and *NP* genes from all human and animal samples, using nested PCR. For long *GP* fragments (2,031 nt), the first round (55°C) was amplified with sense primer 5′-GTGAGCGTAATCTTCATCTC-3′ and antisense primer 5′-TTGTTCAACTTGAGTTGCCT-3′, and the second round (55°C) was amplified with sense primer 5′-CAAGGAAGGGAAGCTGCAG-3′ and antisense primer 5′-GAATCACATTGGCTATGTTT-3′. For short *GP* fragments (538 nt), the first round (54°C) was amplified with sense primer 5′-GAAGGTGTCGTKGCATTTCTGAT-3′ and antisense primer 5′-CCTTGAYTGTGCACTTGAACCA-3′, and the second round (60°C) was amplified with sense primer 5′-TGAGAGAGCCGGTCAATGCAAC-3′ and antisense primer 5′-GAGGAATTTTCTGAAGCCATG-3′. The *NP* gene from human and animal samples was amplified in two fragments: first (55°C) by using sense primer 5′-TTTGCAAGTCTATTCCTTCCG-3′ and antisense primer 5′-CCGTTTTCCGAGTAACTCT-3′ and second (55°C) by using sense primer 5′-GATCCGACTGACTCACAGGATA-3′ and antisense primer 5′-GGCTCATCCTTCATCATATGAT-3′.

Phylogenetic Analyses. Data sets used for the analyses included 21 sequences of the complete *GP* gene (2,031 nt), 24 sequences of partial *GP* (538 nt), and 18 sequences of the partial *NP* gene (1,448 nt). All previously published ebolavirus sequences were included in the analyses, and ebolavirus-Reston, -Sudan, and -Ivory Coast were included as outgroups to root the ZEBOV trees. For *GP*, sequences were translated and aligned at the amino acid level by using the Clustal W algorithm implemented in the MegAlign program (Lasergene software; DNASTAR, Madison, WI) before converting data back to nucleotides. The same approach introduced no gaps in the *NP* alignment.

Phylogenetic relationships were determined by using Bayesian and maximum likelihood (ML) methods. Adequate substitution models were selected based on Akaike's information criterion from a broad suite of evolutionary models (21, 22), using BASEML in PAML (23) and PAUP* 4.0b10 (24). The best model for both genes was a codon position (CP) model (22), which distinguished two partitions, the first of which grouped all sites in the first and second CP and the second of which contained all sites in the third CP. Each partition also had its own transition/transversion ratios, base frequencies, γ-rate distributions, and relative rates. This model was implemented in all analyses if possible. However, CP models are currently not

supported by programs that use ML to estimate phylogenies. Therefore, all ML analyses were carried out by using the next best non-CP model.

Bayesian analyses were conducted with MrBayes software, Version 3.1 (25), using two independent Markov Chain Monte Carlo runs with 10 million generations each, sampling trees every 1,000 generations and with a burn-in of 1,000 trees. A final standard deviation of <0.01 for the split frequency was taken as an indication that convergence had been achieved. ML analyses were carried out with PAUP* by using a heuristic search algorithm. Bootstrap proportions were obtained from 1,000 replicates.

We performed two types of analysis in PAUP* to examine whether the data provided evidence for recombination among different ZEBOV lineages. First, we tested whether the tree topologies for *GP* and *NP* were different when considering only outbreaks that are common to both trees. Unrooted ML trees of all ZEBOV sequences (i.e., excluding the outgroup) were found for each of the two genes, and isolates represented in just one of the data sets were pruned from the tree, resulting in both trees containing sequences from the same set of 14 isolates. Trees were then compared by using the Shimodaira–Hasegawa test. Second, we formulated topological constraints based on the relative position of the three groups (A, B, and R) in the phylogenies. In the *GP* tree, for example, group R formed a clade that was nested with a larger clade together with group A (Fig. 1*A*). Enforcing this topological constraint while heuristically searching for the ML tree for *NP* produced a tree that could subsequently be compared with the original ML tree with a Shimodaira–Hasegawa test. In the *NP* tree, group A formed a clade (Fig. 1*B*), providing the corresponding topological constraint for the analysis of *GP*.

The age of the split between the two ZEBOV lineages and other tree nodes was estimated with BEAST software, Version 1.4 (26), using only ZEBOV sequences and their dates of isolation. Implementing the aforementioned CP model, the analysis in BEAST was run under a constant population size coalescent model for 11 million states, of which the first 1 million were removed as burn-in. Estimating parameters under an exponential growth model produced virtually identical results (data not shown).

The partial *GP* and *NP* sequences obtained in this study are listed in *SI Materials and Methods*.

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